TOPICAL BIO(IN)EQUIVALENCE OF METRONIDAZOLE FORMULATIONS *IN VIVO*

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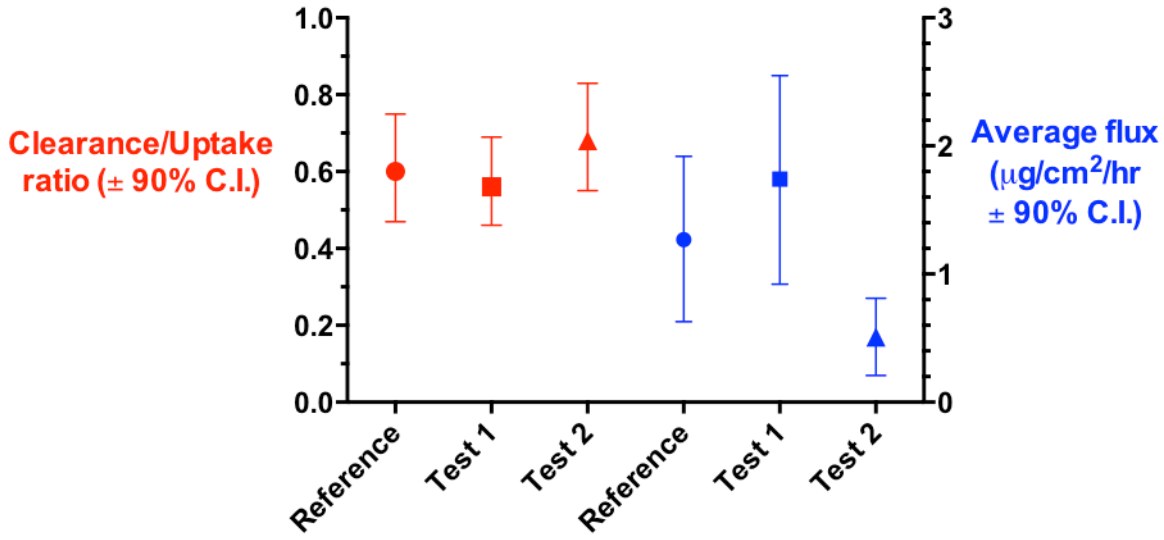
Graphical abstract



ABSTRACT

The topical bioavailabilities of metronidazole from a commercially available 'reference' product (Rozex®) and two extemporaneous test formulations were compared. With the reference drug product, a full skin pharmacokinetic profile, *in vivo* in human volunteers (following a 6-hour uptake and clearance over the subsequent 22 hours), was obtained using an improved stratum corneum (SC) sampling procedure. Then, a two-time point SC sampling method enabled the bio(in)equivalence of the test formulations to Rozex® to be evaluated. One test formulation was shown to be bioequivalent to Rozex®, both for uptake and clearance, whereas the other (more viscous and less spreadable) formulation was not. The delivery of metronidazole into the underlying viable epidermal tissue from Rozex® and from the equivalent test formulation was 2.5 to 3.5-fold higher than that from the inequivalent extemporaneous vehicle. The results highlight that the quantitative composition of a formulation, as well as its physical properties that influence events that take place at the vehicle-skin interface, can have a dramatic impact on the delivery of drug into the SC and subsequently to the viable skin layers below. The reproducible, sensitive and facile *in vivo* methodology employed may prove of particular value where regulatory approval of generic formulations lacks objective rigour.

Keywords: topical bioavailability; topical bioequivalence; skin; metronidazole; stratum corneum sampling *in vivo*; skin pharmacokinetics

1. Introduction

The development of methodology, both *in vivo* and *in vitro*, to determine the bioavailability of topically applied drugs, the site of action of which is on or within (or even just below) the skin, is the subject of considerable attention at the present time (Yacobi, *et al.*, 2014). A particular driving force for this level of interest is to establish reliable and validated approaches to assess the bioequivalence between topical drug products so that less expensive generic formulations can gain regulatory approval and lower the burden on healthcare budgets.

Currently, there is no standardised methodology for topical bioavailability or bioequivalence measurement, and different regulations apply in different countries. Most typically, the approaches adopted by the U.S. Food & Drug Administration (FDA) are dominant and are followed by other agencies such as the European Medicines Agency (EMA) and the U.K. Medicines and Healthcare products Regulatory Agency (MHRA). Specifically, in the majority of cases, clinical studies, which are usually expensive, prolonged and poorly discriminating, are required to establish bioequivalence (Shah *et al.*, 1998; Braddy *et al.*, 2015); a particular exception involves corticosteroid formulations, for which the vasoconstriction assay may be used (US FDA, 1995), and a few other exceptions have been granted for specific products (e.g., acyclovir ointment, a lidocaine patch and dapsone and ivermectin products) (Draft Guidance on Acyclovir, 2012; Draft Guidance on Lidocaine patch, 2016; Draft Guidance on Dapsone, 2017; Draft Guidance on Ivermectin products, 2017). Other countries have also adopted FDA standards but some, like Japan and South Africa, have also accepted the surrogate *in vivo* technique of stratum corneum (SC) sampling using tape-stripping (Braddy *et al.*, 2015). In contrast, elsewhere, there exists essentially no requirement for the establishment of *in vivo* bioequivalence of topical products. For example, in Brazil, the principal requirements for the registration of a generic product are (a) pharmaceutical equivalence, and (b) that the composition of the generic formulation should contain excipients with the same function as those in the reference product (Brazil, 2011).

In addition to SC sampling, other techniques being examined closely as surrogates (either alone or in combination) for clinical trials are *in vitro* skin permeation experiments and *in vivo* microdialysis, including open-flow microperfusion (Bodenlenz *et al.*, 2017; Yacobi, *et al.*, 2014). The former, of course, has been widely used in product development (both topical and transdermal) for many years and now seems likely – at least in some form – to be eventually recognised as a regulatory tool.

While recent data from open-flow microperfusion experiments appear to indicate a real step-change in the quality of microdialysis data (Bodenlenz *et al.*, 2017), there remains much to be done before one can envisage this technically highly-demanding approach as a routine method.

SC tape-stripping has had a chequered past, an FDA draft guidance having been withdrawn relatively quickly after its publication because of inconsistency in the results from two very qualified laboratories (US FDA, 1998). Further, despite a clear diagnosis and understanding of why this happened, in addition to well-supported demonstrations of the usefulness of an improved protocol (N'Dri-Stempfer et al., 2009) to distinguish bio(in)equivalence between anti-fungal (econazole) and non-steroidal anti-inflammatory (diclofenac) formulations (Cordery et al., 2017), the SC sampling method has yet to regain the confidence of the FDA, or of those regulatory agencies which follow its lead.

Nevertheless, the SC represents an accessible and easily interrogated skin compartment *in vivo*. The recent results from the diclofenac study (Cordery *et al.,* 2017), and their correlation with *in vitro* skin penetration data, demonstrate that the technique also has value for assessing the bioavailability of drugs with sites of action not only in the SC (such as econazole (N'Dri-Stempfer *et al.,* 2009)), but in skin layers below the barrier as well. For this reason, the improved tape-stripping method (N'Dri-Stempfer *et al.,* 2009) has been used in the research reported here which aimed to evaluate the bioequivalence (or not) of two extemporaneous metronidazole formulations to the marketed Rozex® product, the only topical formulation of this drug available in many countries (including Brazil). The 'generic' formulations contained the same concentration of metronidazole and the same excipients, but differed in their spreadabilities and viscosities from Rozex®. It follows that, in terms of the FDA's definitions (Chang *et al.,* 2013), the test formulations were Q1 (having the same components) with Rozex®, but not Q2 (i.e., same amounts of the same components) or Q3 (same amounts of the same components arranged in the same way).

2. Materials and methods

2.1 Human subjects

28 healthy volunteers, 19 females and 9 males, participated in the study. The mean (range) age, weight, and height of the subjects were 24 (21-32) years; 62.2 (53-78) kg; and 165 (156–175) cm, respectively. The study protocol (CAAE 34657814.2.0000.5208) was approved by the local ethics committee of the Universidade Federal de Pernambuco, Recife, Brazil. The subjects provided their informed consent prior to participating in the study.

2.2 Materials

Metronidazole was from Hubei Hongyuan Pharmaceutical, Hong Kong, China; Rozex® was purchased from Laboratoires Galderma, Alby-sur-Chéran - France; sodium hydroxide and methyl and propyl parabens were acquired from Vetec, Rio de Janeiro, Brazil; propylene glycol was obtained from Henrifarma, São Paulo, Brazil; and Carbopol was purchased from Fagron, Jundia, Brazil.

2.3 Formulations

Two extemporaneous formulations of metronidazole comprising the same drug concentration and the same excipients as the commercial product (Rozex®) were prepared (Table 1). The two test formulations differed only in the quantity of gelling agent used.

Table 1: *Composition (% w/v) of the extemporaneous formulations defined as Test 1 and Test 2.*

Component	**Test 1**	**Test 2**
Metronidazole	0.75	0.75
Propylene glycol	5.0	5.0
Carbomer	1.0	1.5
Methyl paraben	0.18	0.18
Propyl paraben	0.02	0.02
EDTA	0.05	0.05
NaOH 20%	qs pH 4.0	qs pH 4.0
Water	qs 100	qs 100

The apparent viscosities of the three formulations were determined on 15 g samples using a concentric cylinder-type rheometer (MCR 301, Anton Paar Brasil Ltda, Sao Paulo, Brazil) with ASTM spindle 7 at 30 rpm and 25°C. The spreadability test was performed according to published procedures (Borghetti *et al.,* 2006).

114

115 2.4 Stratum corneum (SC) sampling experiments

116 The principal experimental goal of this work was to determine, using the improved SC tape-stripping
117 approach (N'Dri-Stempfer *et al.,* 2009), that has been validated for econazole (N'Dri-Stempfer *et al.,*
118 2009) and, more recently, diclofenac (Cordery *et al.,* 2017), whether the two extemporaneous
119 metronidazole test formulations were equivalent to Rozex®. The new protocol calls for an assessment
120 of equivalence to be made at one so-called 'uptake' time, and one so-called 'clearance' time, a much
121 less labour-intensive method than that initially proposed in the now-withdrawn FDA draft guidance
122 (US FDA, 1998).

123 To select the most appropriate uptake and clearance times, a preliminary study was first performed to
124 measure drug levels in the SC after a series of different uptake times (1, 2, 4 and 6 hours), and a series
125 of clearance times (2, 6, 11, 14, 18 and 22 hours) post-removal of the Rozex® formulation. These
126 experiments were conducted (on each of the 14 volunteers) following exactly the method of N'Dri-
127 Stempfer *et al.* (N'Dri-Stempfer *et al.,* 2009) with the five specific refinements designed to
128 significantly improve the quality and reproducibility of the data obtained: (a) a rigorous cleaning of
129 excess drug product from the application site at the end of the uptake period; (b) retaining the quantity
130 of drug recovered in the first two tape-strips as material that had been taken up and would ultimately
131 become available in the underlying skin; (c) increasing the number of tape-strips removed to ensure
132 collection of most (> 75%) of the SC; (d) controlling the tape-stripped skin area to avoid 'edge' effects
133 and lateral spread of formulation (N'Dri-Stempfer *et al.,* 2009); and (e) combining tape-strips into
134 groups for drug extraction and subsequent analysis to enhance quantitation.

135 The ventral forearms of the volunteers were first washed with water and gently dried with paper towels.
136 Thirty minutes later, the Rozex® formulation was applied to 10 sites distributed over the two arms (one
137 each for the four uptake times and the six clearance times); another untreated site was also delineated
138 on each arm for tape-stripping to provide an analytical control. Each treated site (2.54 cm^2 in area)
139 was demarcated with a circular template (Scotch Book Tape, 3M Co., St. Paul, MN, USA), and 143.5
140 mg of Rozex® (i.e., 56.5 mg/cm^2) was applied, achieving an even and complete coverage of the skin
141 with the formulation. The sites were then occluded with a 4.9 cm^2 plastic chamber (Hill Top Research,
142 Inc., Ohio, USA) to prevent any loss of the formulation from the skin surface. At each of the designated
143 uptake times (1, 2, 4 and 6 hours), one plastic chamber was removed and the site cleaned of residual
144 formulation with two isopropanol wipes (Biosoma® Laboratorios, São Paulo, Brazil). A smaller
145 template (1.77 cm^2) was then centered over the treated area and the SC was then repeatedly tape-
146 stripped (Scotch Book Tape). A maximum of 30 tape-strips were taken during which regular
147 measurements of transepidermal water loss (TEWL) (Tewameter, Courage & Khazaka GmbH,

Cologne, Germany) were recorded to assess the fraction of SC that had been removed. If TEWL reached more than 6-times the value observed before tape-stripping commenced, no more SC was removed as the barrier had by then been reduced to less than 25% of its normal function (Kalia *et al.*, 1996; 2000).

Further, at 6 hours, all of the 'clearance' sites were exposed and subjected to the same cleaning procedure as described above. The skin sites were then left open to the ambient conditions before being successively tape-stripped at 2, 6, 11, 14, 18 and 22 hours later.

Having conducted this preliminary set of experiments, the bioequivalence protocol was then performed on Rozex® and the two test formulations using 6 hours for both the uptake and clearance times. In this case, after cleaning the volunteers' forearms, formulations were applied to 12 treatment sites (3 formulations per subject, and duplicate applications of each formulation on opposite arms); for each volunteer, on one ventral forearm, the 6 uptake sites to which the formulations were to be applied were randomly assigned between the wrist and the elbow fold; the 6 clearance sites on the opposite arm mirrored those used for uptake in each volunteer. An untreated site was again tape-stripped to provide an analytical control. Application and removal of the formulations, and the tape-stripping procedures, followed exactly the protocol described above except that only one uptake time (6 hours) and one clearance time (6 hours) were considered. Quantitative data on the number of tape-strips removed in the uptake and clearance 'arms' of the study are in Supplementary information, Table S1. Before any tape-stripping in the bioequivalence study, the volunteers were asked to report any adverse effects that they may have experienced, and the treated skin sites were inspected visually by the investigators.

2.5 Metronidazole extraction and analysis

The drug was extracted from tapes 1 to 14 individually by shaking with 1 mL of acetonitrile in a closed vial for 6 hours; tapes 15-17, 18-20, 21-23, 24-26 and 27-30 were grouped for extraction, and drug was extracted therefrom in the same way. Following filtration, the extraction samples were analysed for metronidazole with a previously validated high-performance liquid chromatography method with UV detection at 320 nm (Shimadzu Corp. (Kyoto, Japan) (Melo, et al. 2016). Separation was performed on a C18 reversed-phase column 150 x 4.60mm and a C18 (5µm) pre-column 4 x 4 mm (5 µm) (Shimadzu Corp.) at 35°C. The mobile phase was an 88:12 mixture of 20 mM monobasic sodium phosphate buffer at pH 3.0 and acetonitrile; the flow rate was 1 mL/min, the injection volume 20 µL.

2.6 Interpretation of results

A non-compartmental analysis method was used to analyse the results from the preliminary SC sampling experiments (Phoenix WinNonlin Professional version 5.0, Certara, Princeton, NJ, USA). From the profiles of the quantity of metronidazole in the SC as a function of time, the following

181 'conventional' pharmacokinetic parameters were determined: (a) The maximum quantity of drug in
182 the SC (A_{max}) was directly observed from the data. (b) The rate constant describing metronidazole
183 elimination from the SC (k_e) was determined from the slope of the linear regression of the 'clearance'
184 phase of the log-transformed drug quantity *versus* time profile; the corresponding elimination half-life
185 was found from $t_{1/2} = \ln 2/k_e$. (c) The area under the SC quantity of drug profile as a function of time
186 ($AUC_{0-\infty}$) was calculated using the trapezoidal method for that portion up to the last measured value
187 (A_t) and the standard extrapolation for that part from the final measurement to $t = \infty$, i.e., $AUC_{0-\infty} =$
188 $AUC_{0-t} + A_t/k_e$.

189 Analysis of the results from the bioequivalence protocol followed the published approach of N'Dri-
190 Stempfer *et al.* (2008); briefly, a test formulation (Test 1 or Test 2) was considered bioequivalent to
191 the reference Rozex® product if the ratio (± the 90% confidence interval) of the amount of drug in the
192 SC from the test product to that from the reference formulation was within the range of 0.8 to 1.25.
193 Determinations of bioequivalence (or not) were performed using (i) the drug amount in the SC after
194 the 6-hour uptake period, and (ii) the quantity of metronidazole in the SC following the subsequent 6
195 hours of clearance. Although the sum of the SC levels determined in uptake and clearance has also
196 been reported as an additional metric in previous work (N'Dri-Stempfer *et al.*, 2008), there appears to
197 be no clear mechanistic justification for doing so and such calculations have not been performed on
198 the data obtained in this study.

199

3. Results

3.1 Formulation characteristics

The measured physical properties of the test and reference (Rozex®) formulations are in Table 2.

Table 2: *Physical characteristics of the test and reference formulations studied (mean ± S.D.; n = 6)*

Formulation	Test 1	Rozex®	Test 2
pH	4.40 ± 0.03	4.59 ± 0.05	4.15 ± 0.07
Viscosity (Pa.s)	19.9 ± 0.33	22.2 ± 0.40	28.8 ± 0.63
Spreadability (cm^2)	4.03 ± 0.12	2.59 ± 0.02	0.78 ± 0.07

The two test formulations differed only in the quantity of gelling agent; that is, the products could be considered, using the U.S. F.D.A terminology, as Q1 equivalent (same components), but Q2 inequivalent (same components but not in the same quantities). The higher quantity of Carbomer in Test 2 led to a more viscous and less spreadable formulation than Test 1; the values for the two test vehicles bracketed those of the reference product.

3.2 *In vivo* tolerability

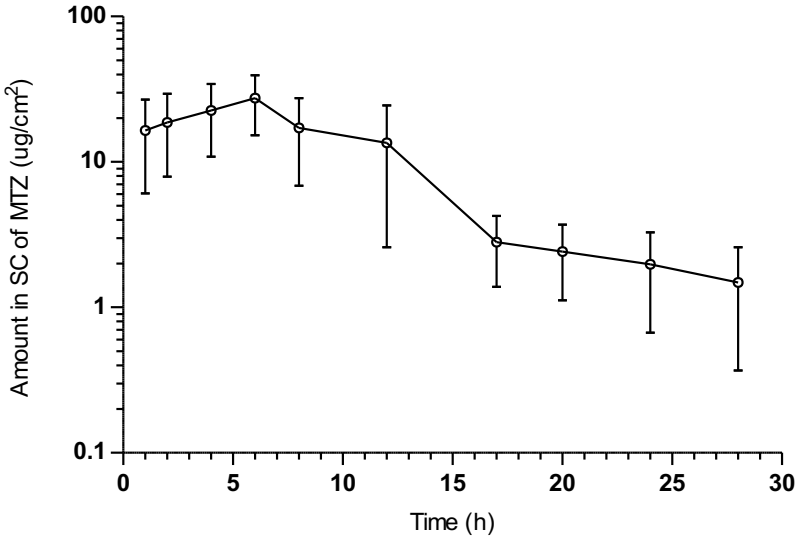
The distribution of skin types amongst the volunteers was: 3 of type II, 4 type III, 4 type IV and 3 type V. After 6-hour exposure to the formulations, no visible signs of irritation were observed in any volunteer. Similarly, at the 'clearance' sites, no redness at the treated skin sites had developed before SC sampling. Post-tape-stripping at both uptake and clearance sites, the skin was visibly irritated. However, the intensity of the reaction was no different at the control, untreated tape-stripped sites. Nonetheless, all volunteers fully completed the experiment.

3.3 SC sampling *in vivo*: pharmacokinetic profile and bioequivalence assessment

The results of the preliminary series of experiments are summarized in Figure 1, which presents the average profile (derived from 14 subjects) of the quantity of metronidazole in the SC as a function of time over 28 hours. In this period, drug uptake was measured at 4 times over the first 6 hours and drug clearance was determined on 6 occasions over the subsequent 22 hours. As expected, the maximum amount of drug found in the SC (A_{max}) was achieved after the longest uptake time (i.e., 6 hours); the mean (± S.D) value of A_{max} was 27.7 (±10.1) μg/cm^2. Assuming a first-order clearance of metronidazole from the SC after the longest uptake time of 6 hours, linear regression of the log-transformed drug quantity *versus* time profile between 6 and 28 hours yields the average (± S.D, n = 14) value for the elimination rate constant (k_e) of 0.14 (±0.03) h^{-1}; the corresponding half-life is therefore 5.1 (± 1.0) hours. The average r^2 value of the 14 log-linear regressions was 0.90 with a standard deviation of 0.04. The mean (± S.D) measured area under the SC amount of drug *vs.* time

profile ($AUC_{0\text{-}28h}$) was 288 (±133) (µg•h)/cm^2 and, using the calculated k_e, $AUC_{0\text{-}\infty}$ was determined to be 299 (±135) (µg•h)/cm^2.

Figure 1: *Kinetic profile of the quantity of metronidazole in the stratum corneum in vivo during uptake ($t \leq 6$ hr) and clearance ($t \geq 6$ hr) phases following topical application of Rozex®. Each data point represents the mean (± S.D.) value from 14 volunteers.*



The results of the subsequent bioequivalence protocol are summarised in Table 3 and Figure 2. An analysis of variance followed by multiple comparison tests when appropriate indicates clearly that, while the average values of drug quantities in the SC (both in uptake and clearance) are not significantly different between the reference product and formulation Test 1, there is a significant difference between the reference and formulation Test 2. However, the ratios of drug amount in the SC in clearance to that in uptake did not differ significantly between the formulations.

When the ratio of drug quantity in the SC following application of a test formulation to that after treatment with the reference product is determined during uptake and clearance, the results expressed as the mean values and the 90% confidence intervals are as shown in Figure 3. Traditionally, as used by the US FDA, for example, the average ratio and the 90% confidence limits must fall within the range 0.8 – 1.25 for a generic product to be considered equivalent to the reference (US FDA, 2007). It follows from the results in Figure 3, therefore, that formulation Test 1 was found to be bioequivalent from the data for uptake and for clearance. In contrast, formulation Test 2 was clearly inequivalent for uptake and clearance.

Table 3: *Results of the two-point SC sampling bioequivalence protocol in vivo. The amounts of metronidazole measured in the SC (geometric mean, and 90% confidence interval (C.I.); n = 14) during uptake and clearance, together with the corresponding ratios of clearance-to-uptake for the reference and two test products tested.*

Formulation		Reference	Test 1	Test 2
Mass of Drug at uptake (µg/cm^2)	Average	17.7	20.4	7.8
	Lower 90% C.I.	14.5	16.2	6.1
	Upper 90% C.I.	21.8	25.7	9.9
Mass of Drug at clearance (µg/cm^2)	Average	10.6	11.6	5.3
	Lower 90% C.I.	8.6	9.4	4.4
	Upper 90% C.I.	13.3	14.3	6.5
Clearance/Uptake ratio	Average	0.60	0.57	0.70
	Lower 90% C.I.	0.48	0.46	0.57
	Upper 90% C.I.	0.76	0.70	0.86

Figure 2: *The quantities of metronidazole measured in the SC (mean ± the upper and lower 90% confidence interval; n = 14) during uptake and clearance following application of the reference and test formulations.*

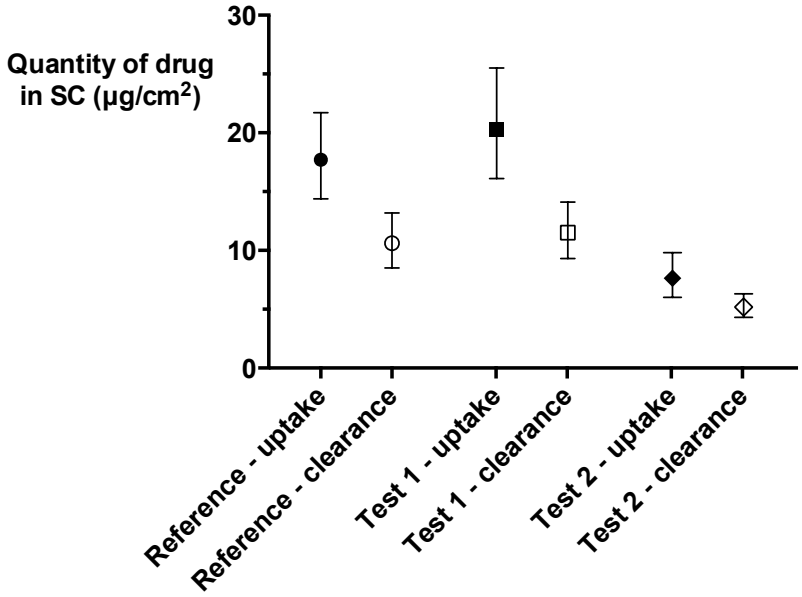
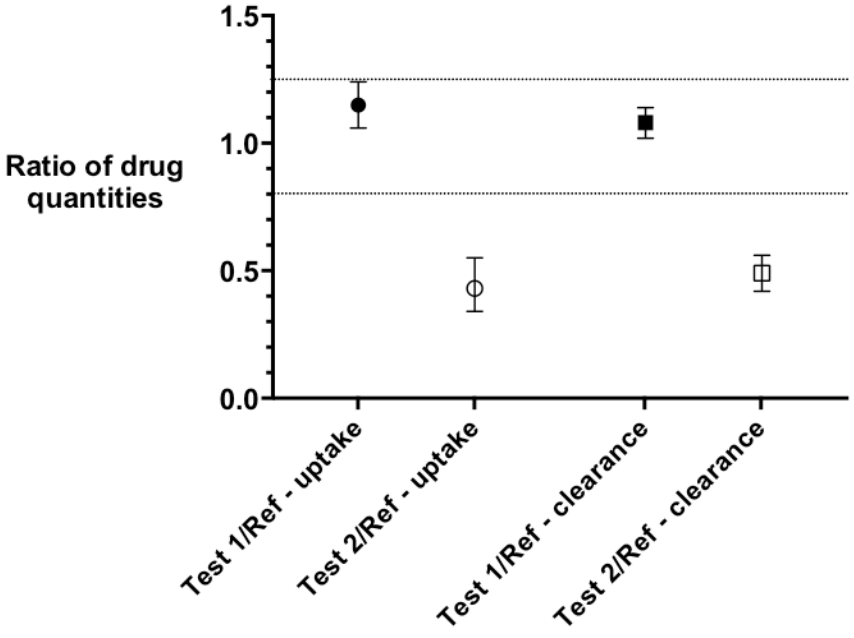


Figure 3: *Bioequivalence assessment of the extemporaneous gels compared with the reference listed product (Rozex®). The ratios (mean ± the upper and lower 90% confidence interval; n = 14) were determined using the quantities of drug in the SC during uptake and clearance. The 0.8 to 1.25 range for the ratio, for which bioequivalence is implied, are indicated on the graph.*



4. Discussion

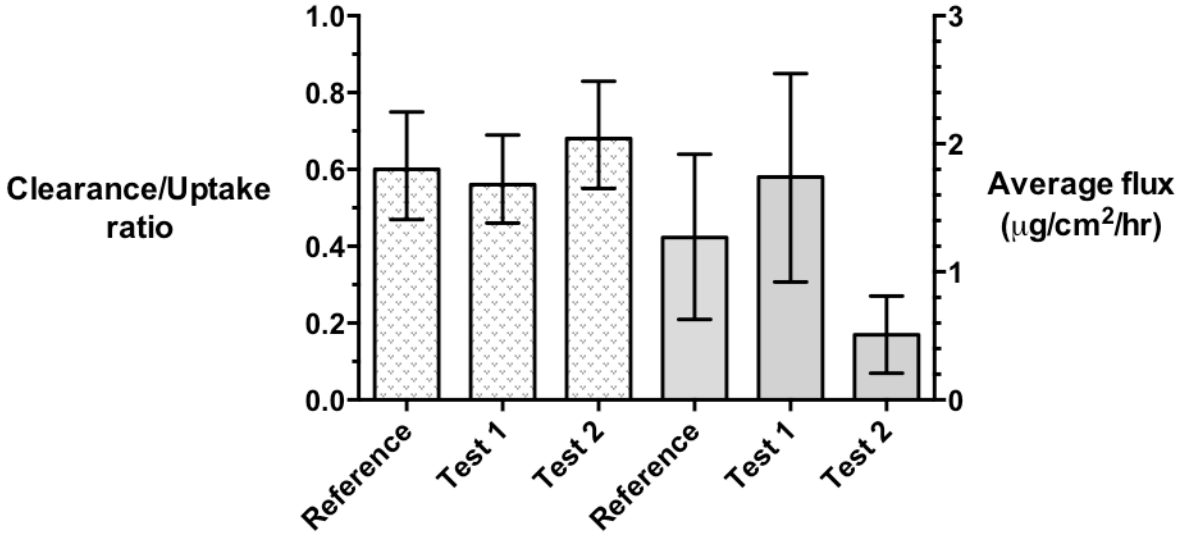
Measurement of the physical characteristics of the two test formulations confirms that their Q2 inequivalence to Rozex® translates into evidence of Q3 inequivalence as well. A one-way analysis of variance followed by Tukey's multiple comparison test reveals that the pH, viscosity and spreadability values of each of the two test formulations differ significantly ($p < 0.05$) from each other and from those of Rozex®, the reference product.

The preliminary SC sampling protocol produced a classic pharmacokinetic profile that adequately characterised the uptake and 'clearance' of metronidazole from this skin compartment after application of Rozex®. This type of information, derived using the improved and previously validated SC sampling methodology (N'Dri-Stempfer *et al.*, 2008), was effectively the intention of the original F.D.A. draft guidance on 'dermatopharmacokinetics' (or DPK) (US FDA, 1998). Clearly, however, with respect to using the approach for the assessment of bio(in)equivalence between different formulations, the protocol is extremely labour-intensive in terms of the sample handling and analytical chemistry involved (Pershing et al., 2001).

It was for this reason that the simplified two-time point method was developed and successfully applied to the comparison of three econazole formulations (N'Dri-Stempfer *et al.*, 2009). In the bioequivalence set of experiments reported here, this protocol (using uptake and clearance times of 6 hours) again generated reproducible data and differentiated between Rozex® and the two test formulations with a modest number (i.e., 14) of subjects (Table 3 and Figure 2).

First of all, the clearance/uptake ratios across the three formulations were consistently around 0.6 (and not statistically different from one another) suggesting that the chosen timings for the two SC sampling events were well-chosen based on the preliminary experiment (Figure 1); that is, the uptake time ensured a significant presence of metronidazole in the SC after 6 hours, while the 6-hour clearance was sufficient for the drug level to have measurably decreased (by nearly 50%, consistent with the results from the preliminary experiment) but yet still be present in an amount well above the analytical limit of quantification. Furthermore, it has been shown that the {log(clearance/uptake ratio)} is proportional to the lag time for clearance (N'Dri-Stempfer et al. 2008, 2009). Therefore, the fact that the clearance/uptake ratios were essentially identical for the three products (as shown in Figure 4) indicates that the clearance rate constant from the SC is the same (i.e., the lag time and drug diffusion rates are similar).

Figure 4. *Clearance-to-uptake ratios (stippled bars, left-hand axis; mean ± the upper and lower 90% confidence interval; n = 14) of metronidazole delivered into the skin from reference and two test formulations; and the corresponding estimated average fluxes (J_{av}) of the drug (shaded bars, right-hand axis; mean ± the upper and lower 90% confidence interval; n = 14) from the SC into the underlying viable skin tissue.*



Second, using the uptake and clearance results, together with the corresponding 90% confidence intervals, it was then possible to undertake a conventional bioequivalence assessment of the two test formulations against Rozex® (Figure 3). It is evident from this analysis that the Test 2 formulation is inequivalent to the reference product, regardless of whether the evaluation is performed using the uptake data or the clearance results. In contrast, the Test 1 formulation was equivalent to Rozex® based on either uptake or clearance data.

Third, it is apparent that the small difference in composition between the Test 1 and Test 2 formulations (Table 1) can have a profound effect on topical bioavailability. Indeed, following the approach described in a recent publication (Cordery *et al.,* 2017), the uptake and clearance amounts of the drug in the SC can be used to estimate the average flux (J_{av}) of metronidazole from the SC into the underlying viable skin tissue (i.e., the site of action):

$$J_{av} = (Q_{Up} - Q_{Cl})/\Delta t \qquad (1)$$

where Q_{Up} is the mass per unit area of drug in the SC at the end of the 6-hour uptake period, Q_{Cl} is the mass per unit area of metronidazole in the SC 6 hours after removal of the formulation, and Δt is the time elapsed between the uptake and clearance measurements (i.e., in this case, 6 hours). The mean values (and lower, upper 90% confidence intervals) of J_{av} for Rozex®, Test 1 and Test 2, calculated from the data in Table 3, are 1.27 (0.63, 1.92), 1.74 (0.92, 2.55) and 0.51 (0.21, 0.81) μg/cm^2/hr, respectively (Figure 4). In other words, metronidazole delivery into the viable skin from formulation Test 1 was >3-fold greater than that from Test 2 (significantly different with $p < 0.01$), a clear reflection

328 of the differential quantities of metronidazole taken up into the SC rather than any difference in drug
329 diffusion through the barrier (as seen by the consistency of the clearance-to-uptake ratios in Table 3).

330 In terms of the significance of these findings, perhaps the most important is that, in countries such as
331 Brazil, the extemporaneous formulations studies here - being Q1 equivalent to Rozex® - would in
332 theory be approvable generics despite, in the case of Test 2, clear inequivalence in terms of drug
333 delivery to the skin. At the very least, therefore, studies such as the one presented here, offer a
334 relatively straightforward *in vivo* methodology with which to compare the local bioavailability of a
335 topical drug administered in a new formulation with that from the reference product.

336 Finally, it is worth pointing out that this research, like almost all recent efforts to address the issue of
337 topical bioavailability/bioequivalence, has involved the single application of drug products to the skin.
338 However, the treatment of major skin diseases involves repeated, chronic dosing and it may be argued,
339 therefore, that it would be better to assess topical bioavailability/bioequivalence under multidose
340 conditions (Wagner, 2013). This is particularly important for formulations which contain excipients
341 that may exert a cumulative effect on skin barrier function. Further work designed to examine this
342 issue in more detail is clearly warranted.
343

5. Conclusions

The delivery of metronidazole into the skin from a commercially available product, and from two extemporaneous formulations, was assessed by an improved stratum corneum (SC) sampling procedure *in vivo*, in healthy human volunteers. While the components of the three formulations were the same, the quantitative compositions, as well as their physical characteristics (including viscosity and spreadability) were different. It was shown that the uptake and clearance of the drug from one of the 'test' formulations were not significantly different from those of the 'reference' product. In contrast, the other 'test' formulation was clearly inferior to the 'reference'. Simple manipulation of the SC sampling data permitted the flux of metronidazole into the underlying viable skin compartment to be deduced; consistent with the bioavailability assessment, the rates of drug delivery from the test formulations were significantly different.

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Supplementary information

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Title: Topical bio(in)equivalence of metronidazole formulations in vivo

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Table S1

Number of tape-strips removed in the uptake and clearance 'arms' of the bioequivalence protocol.

No. of subjects (N) and % of subjects from whom less than 30 tape-strips were taken in uptake and clearance 'arms' of the bioequivalence study						
	UPTAKE			CLEARANCE		
	T1	REF	T2	T1	REF	T2
N	4	5	4	8	7	7
%	29	36	29	57	50	50
Individual no. of tape-strips collected from these individuals						
	UPTAKE			CLEARANCE		
	T1	REF	T2	T1	REF	T2
	21	22	19	18	17	13
	23	25	20	17	16	14
	26	25	24	15	19	19
	22	23	25	19	20	22
		19		20	18	18
				18	25	15
				19	15	22
				20		
Mean	23.0	22.8	22.0	18.3	18.6	17.6
S.D.	2.2	2.5	2.9	1.7	3.3	3.7
%CV	9.4	10.9	13.4	9.1	17.8	21.0

In neither uptake nor clearance was there any obvious difference between products in the number of tape-strips removed for those subjects requiring less than 30 strips to remove the bulk of their stratum corneum. Therefore, none of the excipients (alone or in combination) used in the three formulations are believed to undermine the cohesivity of the skin barrier (as has been reported in other situations – see, for example, Cordery *et al.*, 2017).